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BIODETECTION OF MICROBIAL CONTAMINATION IN PROCESSED FOODS: THE POTENTIAL FOR DEVELOPMENT OF ODOROUS EXCRETORY MATERIALS IN ANIMALS FED RATIONS CONTAINING PYRIDINE-N-OXIDES

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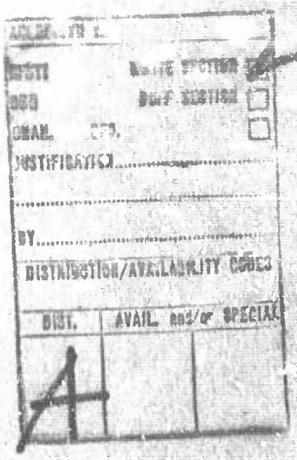

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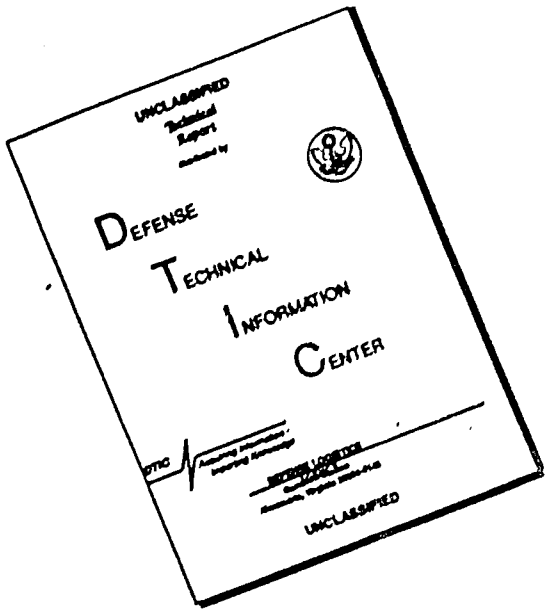

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The objective of the present study was to determine whether or not sufficient substrate would be present in feces and urine of pigs and dogs fed pyridine-N-oxides in rations to result in the production of detectable pyridine odors when the wastes were disposed of in soil. Analysis of lung gas was also done to determine whether or not either reduced or unreduced substrate was excreted via the breath.

Gas chromatography was used for analysis of the substrate and the end-products in the excretory waste products and respiratory gases. Bacteriological analyses were conducted to determine whether or not consumption of the detector substrates caused changes in the intestinal flora of the experimental animals.

The analytical data are discussed in terms of the study objectives and conclusions are made on the effects of the detector substrates in feces and urine.

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FOREWORD

During the initial phases of the development of the flexible container there was concern that such a conventionally sound food package may be subjected to post-processing abuse which could allow microbial contamination. Furthermore, this contamination might result in a health hazard not necessarily detectable by the consumer. It was conceived that the ultimate in safety could be obtained by adding to the food a substrate capable of being altered by invading microbes to give an unmistakable odor signal. The feasibility of an in-package chemical detector (2-ethylpyridine-N-oxide) of post process microbial contamination was established. The objective of this study was to determine to what extent the ingestion of chemical detectors in typical military ration items by pigs and dogs results in the excretion of odorous or potentially odorous waste products (respiratory gases, urine and feces).

The experimental effort described herein was performed in the Life Sciences Section of the Biological Sciences Division, Midwest Research Institute, Kansas City, Missouri, under Contract Number DAAG 17-70-C-0159. This research was done under Project Number 1J664713D548.

The Project Officer and Alternate Project Officer for the U. S. Army Natick Laboratories were Dr. Durwood B. Rowley and Dr. Herbert A. Hollender, respectively.

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ABSTRACT

Certain pyridine-N-oxides have been advocated for use as biological contamination detectors in processed foods. Through a bioreduction brought about by the presence of bacteria a nonodorous heterocyclic N-oxide substrate is converted to an odorous pyridine compound. The odor is used as a signal indicating contamination.

The objective of the present study was to determine whether or not sufficient substrate would be present in feces and urine of pigs and dogs fed pyridine-N-oxides in rations to result in the production of detectable pyridine odors when the wastes were disposed of in soil. Analysis of lung gas was also done to determine whether or not either reduced or unreduced substrate was excreted via the breath.

Gas chromatography was used for analysis of the substrate and the end-products in the excretory waste products and respiratory gases. Bacteriological analyses were conducted to determine whether or not consumption of the detector substrates caused changes in the intestinal flora of the experimental animals.

The analytical data are discussed in terms of the study objectives and conclusions are made on the effects of the detector substrates in feces and urine.

INTRODUCTION

For some time, Midwest Research Institute has carried on a research program for U.S. Army Natick Laboratories on "Biodetection of Microbial Contamination in Processed Foods." The objective of the study was to develop a bacterial contamination detector system that could be used to determine whether or not a thermally sterilized food pack had become contaminated after processing. The warning system was to be used primarily in foods processed in flexible containers although other uses were not excluded.

At the time these studies were started we believed that the most productive approach was one in which a bacterial substrate could be added to a food and which in the presence of small numbers of bacteria would release a characteristic and unmistakable odor. The limiting specifications for development of the detector substrate were precise and difficult to achieve. For example, the original goal was to develop a substrate that was so susceptible to bacterial enzyme action that as few as 1×10^3 to 1×10^4 cells/gram could produce a detectable odor. At the same time, the substrate should be heat stable; should not alter any food characteristic such as odor, flavor, or color; should be nontoxic; should be susceptible to the action of bacteria of several different species and genera; and should be of such low toxicity that it would be acceptable to FDA as a food additive.

Over 200 compounds were tested and of this number four were found which possessed some, but not all, of the attributes listed above. The compounds considered in subsequent tests belonged to two chemical classes: (1) aliphatic sulfones, and (2) heterocyclic N-oxides.

As testing continued the heterocyclic N-oxides were found to possess more of the desired characteristics than the other group of compounds and two members of this class were found to be uniquely responsive. These two compounds (2-ethylpyridine-N-oxide, 2EPNO; and 4-methylpyridine-N-oxide, 4MPNO) were selected along with two aliphatic sulfones for tests in which the detector substrates were processed (commercially) in five foods used by the military. Both short- and long-term tests were incorporated into the study and the reliability of the detectors to warn against consumption of foods contaminated (postprocess) with five different bacterial species was determined. As many as five different storage conditions were studied for their effect on the system.

We found that regardless of the storage condition imposed (-18°C to 38°C) 2-ethyl-pyridine-N-oxide was more reliable as a contamination detector than were 4-methylpyridine-N-oxide, N-propyl-sulfone or N-butyl-sulfone. The 4-methylpyridine-N-oxide, however, was superior to either of the sulfones. Of the bacteria tested, Escherichia coli and Salmonella typhimurium were the easier to detect and Clostridium botulinum the most difficult.

Early in these storage tests we were discouraged because a weak signal was detected in some uninoculated cans (2EPNO) of beef slices and we thought that pH might have been responsible for a non-biological reduction of the substrate and release of 2-ethylpyridine. Tests indicated, however, that 2EPNO was stable under the temperature-pH conditions of the canning process. Storage of the cans for 6 months at 38°C gave us an answer. Nearly 40% of the inoculated cans were swollen indicating a contaminated product. Bacteriological examination demonstrated the presence of a spore-forming bacillus in the canned product. Transfer of this culture to other cans of uncontaminated food containing 2EPNO resulted in development of weak odors similar to those originally found. It appeared that the contaminant produced a signal early in storage at a time when sufficient growth had not occurred to cause the cans to swell. The presence of the contaminant was detected by the system in foods which we had no reason to suspect were improperly processed.

Following the tests on commercially processed foods, we undertook the research which is reported in the following pages. The purpose of this current study was to determine whether or not problems related to development of highly odorous excretory products would arise when animals consumed rations containing pyridine-N-oxide detector substrate. We used pigs and dogs as test animals and in the following sections we report on the methodology developed for analyses of 2EPNO, 4MPNO, 2EP and 4MP in feces and urine. A technique for collecting and analysis of lung gases is also described. The results of analysis for 2EPNO, 4MPNO, 2EP, and 4MP in the excretory wastes are presented in several tables. Experiments designed to determine whether or not the soil microflora is capable of bioreducing 2EPNO are described and the results of the tests are presented.

The analytical data are discussed in terms of the objective of the study and conclusions are drawn about the effects of heterocyclic N-oxide detector substrates in feces and urine.

METHODS

Test Animals

Both dogs and pigs were used as test animals. The dogs were obtained from a licensed laboratory animal dealer. These animals were conditioned mongrels 6 months to 1 year of age. Six different dogs were used in these studies. Pigs were obtained from a commercial pig farm in the Kansas City area. The animals used in our tests were 20-25 lb in weight and were disease-free. Four males and four females were used in the feeding tests.

Animal Quarters

The dogs were housed in an air-conditioned dog room at the Institute's main building. They were kept in stainless steel metabolic cages having draining pans such that feces and urine samples could be collected.

The pigs were kept in an outdoor pen at the Deramus Field Station (four pigs in a 20 x 20 pen). The pen site was selected so that the animals had shade at all hours of the day. Water was supplied continuously by an automatic waterer. The nature of the soil in the area where the pen was located was such that drainage was rapid, therefore muddy conditions seldom developed.

When the pigs were on test, they were transferred to large metabolism cages placed close to the pen area. By placing drain pans in the bottoms of the cages, we were able to collect fecal and urine samples with little difficulty.

Maintenance Feeds

When they were not on test, the pigs were maintained on Purina Pig Startena fed at the rate of 2 lb/pig/day for 25-40 lb pigs and 3 lb/day as the pigs reached 40-60 lb. When the first group of four animals reached about 60 lb in weight, they could not be easily handled in the cages and they were replaced by a second group of four smaller animals.

The dogs were maintained on Purina Dog Chow and were fed at a rate of about 3/4 to 1.0 lb/dog/day. Two different groups of dogs were also fed in the tests.

Test Feeds

Four kinds of commercially canned foods were used as test feeds. These materials were selected because they closely approximate the kinds of rations for which the use of detector substrates is intended. These four products were:

1. Chicken-a-la-king, Lee Brand (CAK);

2. Sliced beef in barbecue sauce, Silver Skillet Foods (BBQ);

3. Beef stew, Lee Brand (BS); and

4. Boned turkey, Swanson Brand (BT).

Detector Substrates

Two heterocyclic N-oxides were tested in the commercial foods:

1. 2-Ethylpyridine-N-oxide (2EPNO). Upon bioreduction, this compound yields a volatile, easily detected, 2-ethylpyridine (2EP).

2. 4-Methylpyridine-N-oxide (4MPNO). Upon bioreduction, this compound yields a volatile product (4MP) but it is less volatile than 2EP. From previous tests, we have observed that the detectable concentration of 4MP appears to be nearly twice that required for detection of 2EP.

Feeding During Test Phase

The two detector compounds were added to the four test foods at concentrations of 0.05 and 0.1%. Usually, enough food for feeding 3 days was prepared at one time and the material for the second and third day was held under refrigeration (4°-5°C) until used.

The usual feeding program was one in which the animals were placed on test feeds for 3 days before the first samples of feces or urine were collected. Control feces and urine were obtained from animals which were kept on maintenance diet and from animals fed a test ration that did not contain any detector substrate.

Animals which were on test feeds that contained detectors were always transferred back to normal maintenance diets for a least 5 days before they were placed on test with a different food or a different detector substrate.

Collection and Storage of Samples

Because scheduling of the chromatographic equipment did not permit us to analyze samples immediately after collection, both fecal and urine samples were sealed in glass containers and frozen to -20°C. They were then held at this temperature until they could be analyzed.

Samples of respiratory gases were analyzed as soon as collected. These samples (obtained from dogs which had been fed beef stew with 0.1% 2EPNO for 5 days) were obtained by anesthetizing the dogs and obtaining samples of lung gases through a large-bore needle inserted into the trachea. Small subsamples were collected in a 50-ml gas-tight syringe by pulling a small portion of respiratory gas each time the dog exhaled until the syringe was full. After the sample was collected, the syringe was sealed at the needle end and immediately taken to the analytical laboratory where a portion of the gas was analyzed by gas chromatography.

Less than 3 min elapsed from the time the sample was collected until it was analyzed.

Analysis of Samples

Sniff tests: The breath of dogs kept on feed containing detector substrate was "sniffed" to determine whether or not 2EP could be detected. Fecal and urine samples as well as the refrigerated rations containing substrates were also sniffed to see whether or not the reduced N-oxides could be detected.

Gas chromatography: All samples were analyzed with a Bendix 2500 gas chromatograph. This instrument is equipped with dual-flame ionization detectors and matched glass columns (3 ft) packed with 10% carbowax 20 M on 100/120 mesh Chromasorb W (HP). Prepared samples were injected onto the column at a temperature (column) of 100°C. After 3 min, the temperature was increased to 180°C at a rate of 20°C/min. The columns were held at 180°C for 5 min.

After the 5-min hold, the column temperature was increased to 200°C. The column was brought to this higher temperature to remove extraneous materials present in the urine and fecal extracts. In order to prevent ghosting of 2EPNO or 2EP, a water sample was injected while the column was at 200°C.

For analysis, the urine samples were thoroughly mixed, centrifuged to remove particulate contaminates and were analyzed by flame ionization chromatography.

Representative chromatograms of urine from dogs that were on regular feed and on the urine spiked with 2EPNO-2EP are shown in Figures 1 and 2. The estimated limit of detection in urine for the unreduced N-oxides is 5 ppm and for the reduced compounds it is 1 ppm. In the tables of this report, "none" means any concentration below these limits.

Fecal samples were prepared for analysis by extracting a 5-g portion with 10 ml of 1N HCl. The extract was centrifuged to remove particulate material. The supernatant was decanted and the pH of the extract was raised to 8.0 with concentrated NH_4OH. The extract was centrifuged a second time and a portion of the supernatant was used for analysis. In preliminary tests, a recovery of 103% was obtained from a control sample spiked with 1,000 µg (200 ppm) of 2EPNO. The estimated lower limit of detection for 2EPNO in feces was 10 ppm and for 2EP, 2 ppm. In this report all levels below these limits are indicated as "none".

Respiratory gases: Samples of lung gases were analyzed directly without any preparative treatments. Seven to 10 ml of lung gas were analyzed for each animal on test.

Bacteriology: Total aerobic plate counts were made of fecal and soil samples by preparing pour plates of serial dilutions of these

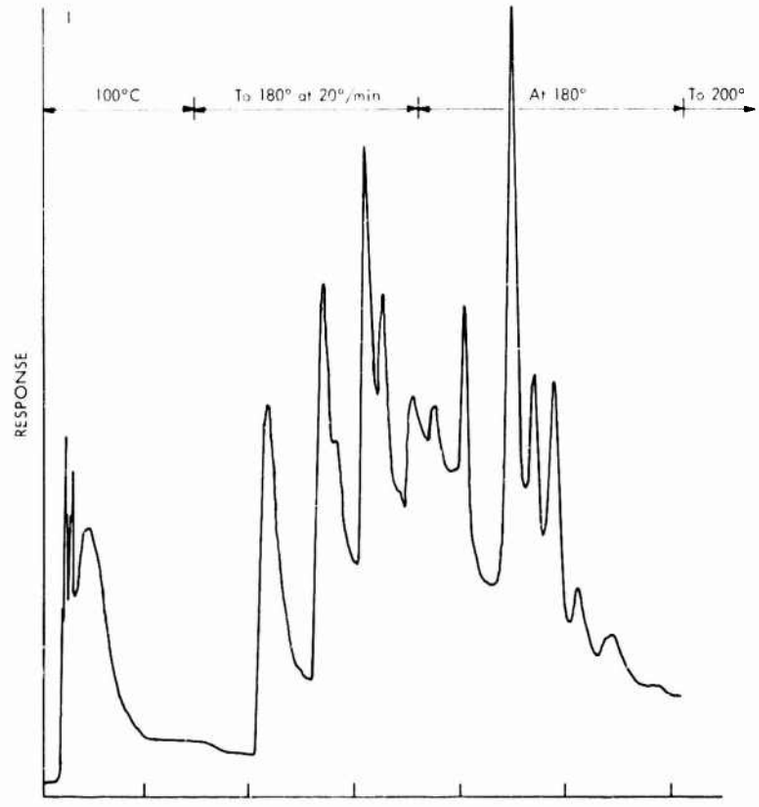

Figure 1 - Gas Chromatogram of Urine From a Dog on Regular Dog Food (2.6 µc urine)

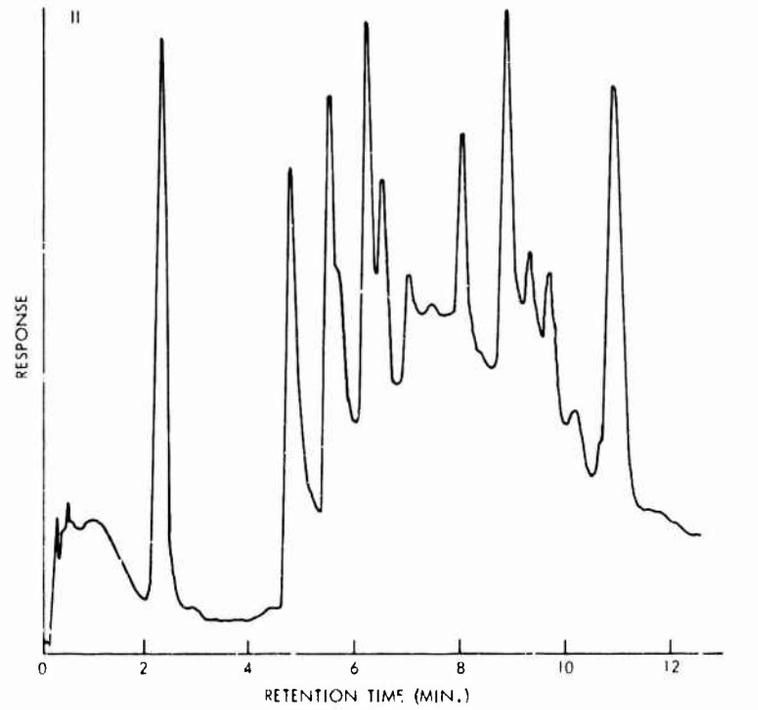

Figure [illegible] - Gas Chromatogram of Normal Dog Urine Spiked With 100 ppm PETN-OH (0.5 µc urine)

materials with trypticase soy agar (with dextrose). Dilutions were made in sterile buffered saline. In some cases, when we wanted to obtain isolates, 0.025-ml quantities of the dilutions were spread uniformly over the surface of the previously poured and dried TSA plates. Coliforms were isolated on violet red bile or MacConkey's agar.

Many of the soil and fecal isolates were tested for their ability to reduce 2EPNO on agar plates. This test was accomplished by streaking isolated cultures on the surface of trypticase-soy agar to which 0.2% 2EPNO had been added. The agar with the N-oxide did not have any detectable odor. However, if the isolate reduced the N-oxide, the presence of 2EP could be readily detected by sniffing.

Reduction of the N-Oxide by Mixed Soil and Fecal Microflora

Several tests were made to determine to what degree soil microflora or fecal microflora would reduce detector material and at what concentration of substrate the reduction would result in strong odors.

In some of these experiments, garden soil was treated with varying concentrations of 2EPNO and 4MPNO and were then held at room temperature in closed containers. Because we were interested only in whether or not the reduction of the compounds could be detected by the nose, we did not run these samples on the gas chromatograph.

A second series of soil tests was performed in which the detector substrate carried in food (chicken-a-la-king) was added to garden soil in closed containers and the mixture incubated up to 5 days.

Other tests were made to determine whether or not feces (from animals fed detector substrate) would liberate 2EP when combined with soil. Concentrations of 2EPNO ranged from 0.025% to 0.8%.

A fourth series of tests was done to determine whether or not feces from normal dogs (on ration without detectors) would accelerate the release of 2EP from soil treated with 2EPNO. Analysis of this test was done by "sniffing" and by chromatographing the gases released on incubation. A fecal sample (7 g) obtained from a dog fed CAK without detector for 3 days was mixed with 100 g of 2EPNO treated soil. The soil was treated with 3 ml of a 2% solution of the N-oxide. The soil-feces mixture was placed in a large serum bottle. The mixture occupied

about one half of the space and the remaining one half was provided for collection of 2EP. The bottle was sealed with a rubber serum bottle stopper which could be easily punctured with a hypodermic needle. After incubation, a sample of the head-space gas was collected with a gas syringe. This sample was analyzed directly without pretreatment by gas chromatography. The stopper was removed from the bottle and the gases in the upper space were sniffed to determine whether or not 2EP could also be detected with the nose.

RESULTS

Fecal samples from pigs which had been fed BBQ, CAK and BS with detector (2EPNO, 0.1%) and from pigs fed BBQ and CAK without detector substrate were plated on TSA to estimate the size of the aerobic bacterial population. We did not attempt to determine the total microbial population because previous tests have shown that the anaercbes do not reduce the N-oxides very effectively, and it was the size of the bioreducing population which was of greatest interest.

The results of the counts shown in Table I appear to indicate that the presence of the detector substrate did not affect the sizes of the populations to any great extent. The ratios of total aerobes to coliform do not appear to be affected much by either the type of food or the presence of the detector.

We were concerned that the presence of the indicator might result in a selection of microbial species which were more readily capable of reducing the substrate than the normal flora, but the results would appear to indicate that changes which occurred were not along species lines.

In order to better characterize the bioreducing populations, we picked isolated colonies from additional agar plates planted with dilutions of fecal material from pigs fed only BBQ and BBQ with detector.

TABLE I

PRESENCE OF BACTERIA IN PIG FECES
CAPABLE OF REDUCING 2EPNO

Ration Fed	Colony Count/G	Coliform/G	% Isolates Which Reduced 2EPNO on Agar Plates
BBQ w/o 2EPNO	5.6×10^6	3.3×10^6	39
BBQ with 2EPNO	2.2×10^6	1.0×10^6	46
BS with 2EPNO	1.6×10^7	7.6×10^6	35
CAK w/o 2EPNO	8.6×10^6	3.8×10^6	20
CAK with 2EPNO	7.9×10^6	3.5×10^6	30

These isolates were then restreaked on MacConkey's and 2EPNO agar. From the responses obtained on these two differential agars, we placed the isolates into groups as shown in Table II. Seven of 18 isolates (39%) from feces of control pigs grew on MacConkey's agar; 83% of the isolates from feces of the test pigs grew on MacConkey's. Whether or not this indicates that coliform types developed to higher percentages of the population in detector treated pigs cannot be established from these limited data but the difference between the two was large. Almost as many of the isolates from the control feces grew on 2EPNO agar as did from test animals. This appears to indicate that for the most part, the presence of 2EPNO is not particularly detrimental to microbial growth.

A greater difference between the two fecal samples was noted when the ability of the isolates to reduce 2EPNO was compared. Forty-one percent (7 of 17) of the control isolates produced a typical 2EP odor from 2EPNO, but 83% of the isolates from test animal feces reduced the N-oxide.

As we have noted in previous reports, certain of the Enterobacteriaceae are very effective reducers of 2EPNO; therefore, we believe that the number of isolates which grew on MacConkey's agar should closely approximate the number of isolates which could reduce 2EPNO agar. If the results reported in Table II are examined, it can be seen

TABLE II

BIOREDUCTION OF 2EPNO AND GROWTH ON MACCONKEY'S AGAR BY BACTERIA ISOLATED FROM PIG FECES

Fecal Samples	Number of Isolates Tested	Growth on MacConkey's Agar	Growth on 2EPNO Agar	Odor Produced on 2EPNO Agar
Pigs fed BBQ w/o 2EPNO	18	7	17	7
Pigs fed BBQ with 0.1% 2EPNO	30	25	30	25

Fecal Samples	Growth on MacConkey's + Odor on 2EPNO	Growth on MacConkey's but no Odor on 2EPNO	No Growth on MacConkey's but Odor on 2EPNO	No Growth on MacConkey's and no Odor on 2EPNO
Pigs fed BBQ w/o 2EPNO	5/7	2/7	2/11	9/11
Pigs fed BBQ with 0.1% 2EPNO	24/25	1/25	1/5	4/5

that there is good agreement between ability to grow on MacConkey's agar and the ability to reduce 2EPNO agar. Of the seven isolates from control feces which grew on MacConkey's, five reduced 2EPNO (71%); 24 of the 25 isolates from test animal feces which grew on MacConkey's agar (96%) also reduced 2EPNO. Eighty-two percent of the isolates from control feces which did not grow on MacConkey's also failed to reduce 2EPNO. The same situation existed for isolates from test animals: 80% of the cultures which failed to grow on MacConkey's failed to reduce 2EPNO agar.

When the results of tests with isolates from both control and test animals fed CAK, BBQ, and BS with and without 2EPNO were combined, it was found that 97% of the fecal isolates which grew on MacConkey's agar reduced 2EPNO and 95% of the isolates that failed to grow on MacConkey's also failed to produce 2EP from 2EPNO agar.

Bioreduction of Pyridine-N-Oxides by Soil Microflora

The results from tests with fecal samples indicated that the presence of 2EPNO at concentrations of 0.1% in animal feeds and 0.2% in agar medium did not inhibit the growth of the normal microflora.

Similar tests were made to determine whether or not the pyridine-N-oxides affected a soil microflora in any demonstrable way. Garden soil was mixed directly with several quantities of 2EPNO and 4MPNO. Various quantities of CAK containing the detector substrates were also incorporated into soil.

It appeared that the presence of neither 2EPNO nor 4MPNO had any great effect on reduction of the soil microflora, Table III. In each case where the detector materials were added alone, a slight increase in population may have occurred.

In the case where 2EPNO was added with CAK, the population appears to have remained unchanged. A bacterial population estimate was not obtained for soil containing 4MPNO in chicken-a-la-king because a very large fungal flora developed in this sample.

When 2EPNO was added to the soil, odor of 2EP could not be detected after 24-hr incubation of any of several concentrations tested (Table IV) and only a slight odor was detected at the 0.8% level after

TABLE III

AGAR PLATE COUNTS ON SOILS HELD
4 DAYS IN SEALED CONTAINERS

Soil Sample	Plate Count (No/G)
Untreated Soil	3.4 x 107
Soil treated with 0.8% 2EPNO	5.2 x 107
Soil treated with 0.8% 4MPNO	8.2 x 107
Soil treated with 0.8% 2EPNO carried in chicken-a-la-king	3.2 x 107

TABLE IV

BIOREDUCTION OF 2EPNO BY THE
MICROFLORA OF GARDEN SOIL

Soil Sample	2EP Odor Present		
	24 Hr	48 Hr	96 Hr
Soil + 0.025% 2EPNO	None	None	None
Soil + 0.05% 2EPNO	None	None	None
Soil + 0.10% 2EPNO	None	None	None
Soil + 0.20% 2EPNO	None	None	Slight
Soil + 0.40% 2EPNO	None	None	Slight
Soil + 0.80% 2EPNO	None	Slight	Strong
Soil + 0.025% 2EPNO in chicken-a-la-king	None	None	None
Soil + 0.05% 2EPNO in chicken-a-la-king	None	None	None
Soil + 0.1% 2EPNO in chicken-a-la-king	None	None	Slight
Soil + 0.2% 2EPNO in chicken-a-la-king	None	None	Slight
Soil + 0.4% 2EPNO in chicken-a-la-king	None	None	Slight
Soil + 0.8% 2EPNO in chicken-a-la-king	None	None	Strong

48 hr. After 96 hr of incubation, a slight odor was detected in soils with 0.2 and 0.4% concentration and a strong odor was detected in the soil with 0.8% detector.

The results from tests in which the detector was added with food (CAK) were similar: no 2EP could be detected in any soil treated with any concentration of 2EPNO up to 48 hr; but after 96 hr, a strong odor of 2EP was present in soil containing 0.8% detector and a slight odor was detectable in soils containing 0.1, 0.2, and 0.4% substrate.

4-Methylpyridine-N-oxide was not as readily reduced by soil microflora as was 2EPNO or the reduced compound 4MP is not as volatile or not as odorous as 2EP. After 96 hr of incubation, the soil samples which contained 0.4 and 0.8% compound developed a slight 4MP odor.

None of the test soils into which CAK with detector was incorporated yielded a 4MP odor. This result is consistent with previous tests with food systems to which pure cultures of bacteria have been added. The reduction of 2EPNO appears to be more readily accomplished by bacteria than is the reduction of 4MPNO.

The results presented in Tables IV and V indicated that unless the detector compounds were present in concentrations that were relatively high, the soil microflora would not bring about reduction enough so that the presence of 2EP and 4MP could be detected. Further, sniff tests performed on each urine and fecal sample indicated that the presence of 2EP and 4MP was not detectable in freshly voided wastes. These two observations suggested that the production of odorous 2EP and 4MP from feces obtained from animals fed the substrates at 0.1% of their diet would be very low; and if the feces were buried in soil, the odors would probably not be detectable.

This supposition (odor development in soils) was tested by incorporating 7-g samples of feces from animals (both dogs and pigs) into 100-g samples of garden soils and after 5 days of incubation in sealed containers checking for the presence of the reduced N-oxides. These tests were done only with samples from animals fed 0.1% 2EPNO, but in no tests were we able to detect the presence of 2EP.

TABLE V

BIOREDUCTION OF 4MPNO BY THE MICROFLORA OF GARDEN SOIL

Soil Sample	4MP Odor Present		
	24 Hr	48 Hr	96 Hr
Soil + 0.025% 4MPNO	None	None	None
Soil + 0.05% 4MPNO	None	None	None
Soil + 0.1% 4MPNO	None	None	None
Soil + 0.2% 4MPNO	None	None	None
Soil + 0.4% 4MPNO	None	None	Slight
Soil + 0.8% 4MPNO	None	Slight	Slight
Soil + 0.025% 4MPNO in chicken-a-la-king	None	None	None
Soil + 0.05% 4MPNO in chicken-a-la-king	None	None	None
Soil + 0.1% 4MPNO in chicken-a-la-king	None	None	None
Soil + 0.2% 4MPNO in chicken-a-la-king	None	None	None
Soil + 0.4% 4MPNO in chicken-a-la-king	None	None	None
Soil + 0.8% 4MPNO in chicken-a-la-king	None	None	None

One additional test was made to prove that the level of substrate present in feces was too low to allow production of 2EP at concentrations which could be readily identified.

Feces from a control dog were incorporated into garden soil and the mixture was treated with 2EPNO. The substrate was added to a concentration equal to 0.056% of the weight of test materials. This sample was incubated in a container in which gases could collect in the head space and be easily sampled. A gas chromatographic analysis of the gas evolved from the soil-feces mixture showed that 2EP had been evolved at a concentration of 103 ng/cc. When the closure was removed from the bottle holding the soil-feces mixture, 2EP could also be detected by sniffing. After a very short period of time, however, the odor could no longer be detected by smelling.

Detection of Reduced Parent Compounds in Feces and Urine of Pigs Fed Pyridine-N-Oxides

The results of analysis of pig feces and urine by gas chromatography are listed in Table VI. These results do not show any kind of consistent pattern; that is, there does not appear to be any trend showing high excretion of 2EPNO or of 2EP in urine. Only in one set of samples (No. 15) did we find both 2EPNO and 2EP in urine and feces. And in this case, there was strong evidence that the samples were contaminated with feed. On the other hand, the results show the concentration patterns are as we thought they should be; i.e., more 2EP in feces than 2EPNO and more 2EPNO in urine than 2EP. Unfortunately, we were unable to collect the total urine and fecal output over the entire feeding period and, therefore, cannot estimate the amount of substrate fed that can be accounted for in the waste products.

It was extremely difficult to feed pigs chicken-a-la-king without contaminating the collection pans with the food; therefore, we are not inclined to consider the values obtained on this one sample as valid.

The results from analysis of samples 4 and 5 appear to be somewhat consistent, but the presence of 2EP may be the result of microbial reduction of 2EPNO during thawing and preparation procedures.

All of the other results appear to indicate that when 2EPNO is present in urine and feces, it is present at a concentration so low that detection of the 2EP which would result from bioreduction would not be easily detected under any conditions except where complete entrapment of released 2EP would be possible.

From the results of studies on bioreduction of 2EPNO and 4MPNO by soil microorganisms, we must conclude that the level of both of the 2 pyridine-N-oxides in feces and urine of pigs would probably be too low to be detectable if these wastes were buried in soil.

Detection of Pyridine-N-Oxides and Products of Bioreduction in Feces and Urine of Dogs

Samples of fecal material and urine were easier to keep free of contamination when dogs were used as test animals than when pigs were used.

TABLE VI

DETECTION OF PYRIDINE-N-OXIDES AND PRODUCTS OF BIOREDUCTION IN URINE AND FECES OF PIGS FED THE OXIDES IN THREE KINDS OF FOODS

Pig Sample Number	Food	Indicator and Concentration (%)	Urine Analysis[a] EPNO or MPNO	Urine Analysis[a] EP or MP	Fecal Analysis[a] EPNO or MPNO	Fecal Analysis[a] EP or MP
1	BBQ	EPNO - 0.05	None	None	None	None
2	BBQ	EPNO - 0.05	None	None	Trace	None
3	BBQ	None	None	None	None	None
4	BBQ	EPNO - 0.1	5.7 ppm	3.3 ppm	None	None
5	BBQ	EPNO - 0.1	9.2 ppm	3.9 ppm	None	None
6	BBQ	MPNO - 0.1	None	None	--	--
7	BBQ	MPNO - 0.1	None	Trace	None	None
8	BBQ	MPNO - 0.5	--	--	None	None
9	CAK	EPNO - 0.1	--	--	None	None
10	CAK	None	None	None	None	None
11	CAK	EPNO - 0.1	None	Trace	None	None
12	CAK	EPNO - 0.1	None	3.4 ppm	None	None
13	CAK	None	None	None	None	None
14	CAK	EPNO - 0.1	9.7 ppm	Trace	None	Trace
15	CAK	EPNO - 0.1	12.1 ppm	3.7 ppm	13.2 ppm	20.6 ppm[b]
16	BS	EPNO - 0.1	None	Trace	None	25.0 ppm[c]
17	BS	EPNO - 0.1	Trace	Trace	None	None
18	BS	None	None	None	None	None
19	BS	EPNO - 0.1	None	None	None	None
20	BS	EPNO - 0.1	None	Trace	None	None
21	BS	EPNO - 0.1	None	None	None	None

a/ In urine none means less than 5 ppm 2EPNO and 1 ppm 2EP. In feces none means less than 10 ppm 2EPNO and 2 ppm 2EP.

b/ Possible contamination by food, see text.

c/ Possible ghosting.

For this reason, we believe that the results of assays shown in Table VII are more representative of the real value for 2EPNO and 2EP in urine and feces than are the data in Table VI.

In most samples, neither 2EPNO nor 2EP were present at concentrations high enough to be detected by gas chromatography.

The foods given to the dogs contained the detectors at 1,000 ppm (0.1%) yet the analysis of feces and of urine indicates that the compounds are present at levels of 1-3 ppm (trace) if detectable at all. We do not know the reason for the decrease (dilution in the gut, utilization of the compounds by microorganisms, etc.) but it is reasonably safe to assume that the levels which are present in feces (2EPNO) would not result in the production of 2EP at concentrations great enough to be easily detected with the nose unless a concentration procedure is used.

Calculations from the results of analysis in Table VII show that 75% of the urine samples did not have detectable levels of 2EPNO; 21% contained a trace (1-3 ppm) and only 4% contained greater than a trace. It was also shown that 86% of the fecal samples did not contain a detectable level of 2EPNO and 14% contained only a trace (7-10 ppm). None of the samples had levels greater than a trace.

Figures almost identical to those calculated for 2EPNO were determined for the levels of 2EP. Eighty-five percent of the urine samples contained no detectable levels and only 12% were found to have a trace. All of the fecal samples (100%) were found to contain no detectable levels of 2EP.

The percentages of fecal and urine samples found to contain each of three levels of 2EPNO, 4MPNO, 2EP and 4MP are shown in Table VIII.

Detection of Reduced Parent Compounds on the Breath and in Respiratory Gases of Dogs Fed 2-Ethylpyridine-N-Oxide

At the time fecal and urine samples were collected from dogs being fed the detector substrates, a "sniff" test was made of the dog's breath. At no time were we able to detect the presence of 2EP or 4MP on the breath of any test animal.

TABLE VII

DETECTION OF PYRIDINE-N-OXIDES AND PRODUCTS OF BIOREDUCTION IN FECES AND URINE OF DOGS

Dog Sample Number	Food	Indicator and Concentration (%)	Urine Analysis		Fecal Analysis	
			EPNO or MPNO	EP or MP	EPNO or MPNO	EP or MP
1	BBQ	EPNO - 0.05	Trace	2 3 ppm	Trace	None
2	BBQ	EPNO - 0.05	Trace	None	Trace	None
3	BBQ	EPNO - 0.05	Trace	None	None	None
4	BBQ	None	None	None	None	None
5	BBQ	EPNO - 0.1	None	None	None	None
6	BBQ	EPNO - 0.1	None	None	None	None
7	BBQ	EPNO - 0.1	None	None	None	None
8	BBQ	None	None	None	None	None
9	BBQ	MPNO - 0.1	None	None	None	None
10	BBQ	MPNO - 0.1	None	None	None	None
11	BBQ	MPNO - 0.1	None	None	None	None
12	BBQ	MPNO - 0.1	None	Trace	None	None
13	Dog food	None	None	None	None	None
14	BBQ	None	None	None	None	None
15	BBQ	EPNO - 0.1	None	None	None	None
16	BBQ	None	None	None	None	None
17	BS	EPNO - 0.1	None	Trace	None	None
18	BS	EPNO - 0.1	5.1 ppm[a/]	3.4 ppm[a/]	--	--
19	BS	EPNO - 0.1	None	None	None	None
20	BS	EPNO - 0.1	None	None	None	None
21	BS	None	None	None	--	--
22	BS	MPNO - 0.1	None	None	None	None

TABLE VII (Concluded)

Dog Sample Number	Food	Indicator and Concentration (%)	Urine Analysis		Fecal Analysis	
			EPNO or MPNO	EP or MP	EPNO or MPNO	EP or MP
23	BS	MPNO - 0.1	None	None	None	None
24	BS	MPNO - 0.1	None	None	None	None
25	BBQ	MPNO - 0.1	None	None	None	None
26	BBQ	MPNO - 0.1	--	--	Trace	None
27	BS	None	None	None	None	None
28	BS	EPNO - 0.1	--	--	None	None
29	BS	EPNO - 0.1	None	None	None	None
30	BS	EPNO - 0.1	--	--	None	None
31	BT	EPNO - 0.1	None	None	None	None
32	BT	EPNO - 0.1	None	None	None	None
33	BT	EPNO - 0.1	Trace	None	None	None
34	CAK	EPNO - 0.1	None	None	Trace	None
35	CAK	EPNO - 0.1	None	None	None	None
36	CAK	None	None	None	None	None
37	CAK	EPNO - 0.1	Trace	None	None	None
38	CAK	EPNO - 0.1	None	None	None	None
		(Respiratory gas)				
39	BS	EPNO - 0.1	None	None		
40	BS	EPNO - 0.1	None	None		

a/ Possible ghosting.

TABLE VIII

PERCENT OF FECAL AND URINE SAMPLES OF DOGS CONTAINING NONE, TRACE OR MORE THAN A TRACE OF PYRIDINE COMPOUND

Pyridine Compound	Sample					
	Urine			Feces		
	0	Trace	> Trace	0	Trace	> Trace
2EPNO	75	21	4	86	14	0
2EP	85	12	3	20	0	0
4MPNO	100	0	0	83	17	0
4MP	88	12	0	100	0	0

We were not sure, however, that the compounds might not be present at levels so low that rapid dilution of the exhaled breath prevented detection. In order to be assured that the compounds were not coming through on the breath of the dogs, we analyzed the respiratory gases by gas chromatography. We accomplished this analysis by collecting lung gases from the trachea of anesthetized dogs. The sample was injected directly into the chromatograph. We assayed gases from a control dog (on beef stew without indicator) and on two samples from a dog that had been fed beef stew containing 0.1% of 2EPNO for 5 days prior to the test.

The results of these analyses confirmed the results of the breath-sniff test: neither 2EPNO nor 2EP were present in the respiratory gases.

DISCUSSION

The primary objective of the research reported here was to determine to what extent the pyridine-N-oxides proposed for use as bacterial contamination detector substrates could be found in waste products of animals. The main emphasis of the study was on analysis of feces and urine for detector substrates and end-products. However, other questions

related to secondary goals were also considered: (1) can the body wastes be disposed of in soil without generation of detectable and specific odors, (2) can foods containing the detectors be disposed of in soil without generation of typical pyridine odors, and (3) will the gut flora be altered by consumption of foods containing the substrates'

The results of analysis of fecal and urine samples by gas chromatography were fairly clear: when either the parent substrate (2EPNO) or the reduced compound (2EP) is present in feces or urine it is at a concentration below that at which it was present in the food (1,000 ppm in food--1 to 25 ppm in feces and urine). The reason for the decrease in concentration is not clear. It may have resulted from dilution by intestinal materials or it may be that the pyridine compound is utilized to some extent by the gut flora.

The results of analyses of urine and fecal samples do not allow us to make conclusions regarding precise routes of excretion of reduced and unreduced substrate. In those cases where fecal or urine samples were found to contain the two compounds, the quantities present indicate that a higher proportion of the N-oxides are excreted in urine (higher than the reduced form) and that the amounts of reduced forms are higher in the feces. This could be expected, however, because the amount of bacterial reduction of the N-oxides by the normal flora in feces would probably be greater than that caused by bacterial contaminants present in the urine samples.

Precise measurements of the physical effects that the detector substrates had on the animals were not made but general observations on the condition of skin and coat quality, alertness, consistency of stools, energy level, etc., did not indicate any detrimental effect on the health of the test animals.

Assessments of kidney damage, liver damage, etc., were not made and since the animals did not consume the test materials over an extended period, conclusions on toxicity of the detectors must be general and qualified.

The results from bacteriological tests appear to indicate that the intestinal microflora contains a higher number of N-oxide reducers than are present in fertile garden soil. This observation is consistent with previous results which have indicated the family Enterobacteriaceae to be very active in bioreduction of heterocyclic N-oxides. This last

observation is strengthened by the correlation which exists between the ability of isolates to grow on MacConkey's agar (selective) and their ability to reduce 2EPNO agar.

When single species were isolated from feces of animals fed BBQ beef slices with and without detector substrate, it was found that all isolates which grew on MacConkey's agar also reduced 2EPNO agar. Over 80% of the isolates which did not grow on MacConkey's agar also failed to reduce 2EPNO agar.

The formulation of MacConkey's agar is such that the Gram-positive organisms are inhibited; therefore, a strong correlation between ability to reduce heterocyclic N-oxides and Gram-reaction can be made indirectly. This correlation may also explain the greater bioreducing capacity of fecal flora compared to the capacity of soil flora.

Evidence that the consumption of foods containing detector substrates altered the composition of the intestinal flora was not strong. The ratio of coliform to total aerobic population in feces did not vary greatly whether or not the animal from which the sample was obtained was fed rations with or without substrate. There was some evidence, however, that the proportion of the normal flora with an ability to reduce 2EPNO did increase in animals fed the substrates for 3 or more days.

CONCLUSIONS

The following conclusions are drawn from an evaluation of the analytical data obtained in this study.

1. Feeding either 2EPNO or 4MPNO at a concentration of 0.1% in foods (which may be used by the military) has no observable effects on the health of pigs or dogs over short feeding periods.

2. The amount of detector substrate (2EPNO-4MPNO) which is excreted in the feces or the urine does not result in development of strong odors of reduced substrate (2EP-4MP) in these waste materials.

3. The disposal of fecal and urine materials from personnel consuming foods containing the contamination detectors should not result in the production of detectable odor when the wastes are dispersed in soil.

4. Foods containing 2EPNO at 0.1% would probably produce detectable 2EP if buried in soil in a concentrated mass but not if diluted prior to burial.

5. Respiratory gases of dogs fed a ration containing 2EPNO did not contain either 2EPNO or 2EP; therefore, development of a highly odorous breath from excretion of 2EP by personnel consuming detector-containing foods is highly unlikely.

6. Consumption of detector-containing foods (2EPNO-4MPNO) should not be expected to alter the normal populations of intestinal bacterial species although greater numbers of the normal flora may exhibit reducing ability in respect to the detector after exposure to the substrate for 3 to 5 days.

SUMMARY

This report is a description of the methods used for analysis and of the results of tests done on feces, urine and respiratory gases collected from dogs and pigs which were fed rations containing pyridine-N-oxides.

The pyridine-N-oxides used in these tests have been advocated for use as detector substrates for indicating the presence of bacterial contaminants in postprocess military rations.

Two detector substrates (2-ethylpyridine-N-oxide, 2EPNO and 4-methylpyridine-N-oxide, 4MPNO) were added to four commercial foods which may be used by the military and these foods were fed to the test animals.

Samples of feces and urine were collected from groups of animals fed the ration both with and without detectors and were analyzed by gas chromatography for the presence of the detector substrates (2EPNO-4MPNO) and their reduced products (2EP-4MP).

Samples of lung gases were collected from dogs by tracheal puncture and were analyzed for 2EPNO and 2E..

Studies were made of feces from control and test animals to determine (1) whether or not feeding the detector substrates altered the intestinal flora of test animals, (2) whether or not feces from test animals developed characteristic 2EP or 4MP odors, (3) to determine whether or not soil had a microflora capable of actually reducing 2EPNO-4MPNO, and (4) to determine whether or not feces and unconsumed rations containing the detector substrates could be disposed of by burial in soil without producing easily detectable odors of 2EP or 4MP.

Analysis of feces and urine by gas chromatography showed that 2EPNO, 4MPNO, 2EP and 4MP were present in these excretory products at concentrations below those fed in the rations (1-25 ppm in urine and feces - 1,000 ppm in foods). The results of these analyses also showed that, even when present, 2EPNO and 4MPNO were at concentrations below the minimum necessary for development of strong odors from bioreduction of the N-oxides. These data indicated that feces and urine could probably

be buried in soil (but should be dispersed) and would not produce easily detectable 2EP.

Foods (garbage) containing the detector substrate at 0.1% would probably yield detectable odors, therefore, foods should not be buried as a solid mass but should be diluted prior to burial or should be well dispersed.

Analyses of the collected lung gas from dogs showed that neither 2EPNO nor 2EP were excreted in the respiratory gases.

The detector substrates did not appear to upset the balance of normal bacterial species in the intestinal tracts of the animals, although when rations were fed containing 2 EPNO for 3 to 5 days the N-oxide supplemented rations appeared to bring about an increase in the numbers of the normal gut flora capable of reducing the pyridine-N-oxides.

From general observations made of both pigs and dogs, consumption of the pyridine-N-oxides for the periods used in these tests (3 to 5 days) did not result in any detrimental effect on the health of the animals.

The conclusions reached from these studies were that (1) detectable odors (by nose) of reduced substrates in feces and urine from personnel consuming detector-containing foods would probably not develop and burial in soil could be used to dispose of body wastes, (2) unconsumed foods would probably have to be diluted with soil or other material before burial to prevent development of strong odors of reduced N-oxide, (3) consumption of the detector-containing rations should not alter the balance of bacterial species in the gut, and (4) the excretion of odorous reduced N-oxides in the breath would not occur.